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A comparison of the acute toxicity and bioaccumulation of boron particles (nano and micro) in *chodatodesmus mucronulatus*

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ABSTRACT

In this study, pigmentation (Chlorophyll *a,* Chlorophyll *b* and carotenoid), bioaccumulation, and oxidative stress response upon boron particles exposure were investigated in *Chodatodesmus mucronulatus*, unicellular algae. The Effects of acute exposure was measured by growing algae in BG-11 media supplemented with varying concentrations of boron particles for 72 h. Nano boron has increased chlorophyll content for 48 h. However, as exposure time was prolonged, nano boron was toxic to the algae. Micro boron particles also indicated similar effects. Nano boron increased chlorophyll *b* at 48 hours. On the other hand, levels of chlorophyll *b* seem to decrease for the remaining part. Bioaccumulation of the boron particles did not notably change upon varying particle concentration. Nano boron particles toxicity is conclusively seen at 72 h of exposure. Additionally, nano boron particles are concluded to be more toxic than micro boron to the *Chodatodesmus mucronulatus.*

1. Introduction

Although the fields nanotechnology and nanoscience have emerged only a few decades earlier, nanomaterials and products at nanoscale have become an established industry due to the extraordinary characteristics of these materials [1]. Increased consumption of nanoproducts resulted in the growing deposits of nano-wastes in certain organisms and environment, which raise the question of human health and environmental pollution [2-4]. These concerns necessitate the investigation of the assessment of nanoparticle toxicity, and thus, the term 'nanotoxicology' emerges. Nanotoxicology is a science discipline that has developed in line with understanding the physico-chemical properties of nano-materials potentially causing toxicity in organisms [5-6]. Nanotoxicology aims to establish a relationship between the physico-chemical properties (for instance; size, surface properties and crystal phase, etc.) and toxicity of nanoparticles (NPs) [7-10]. Great promise of nanotechnology and nanomaterials could underestimate the potential health hazards and corresponding precautinary actions needed to safely use this new technology [11-12]. However, recent studies regarding the toxicology of NPs [13-15] have indicated that NPs could be harmful because of their relatively high specific surface area and unique physico chemical properties. The boron (B) NPs are an example of a nanomaterial that is being used specifically for medical purposes due to its unique property of alpha particle radiation under intense neutron bombardment. Local heating caused by this radiation kills tumor cells and suggested as an effective therapeutical approach to cure cancer [16]. On the other hand, many industrial and urban waste waters and waste water discharges are carried to the rivers, lakes and coastal waters [17]. Evaluation of the aquatic environment is principally assessed by the algae growth inhibition [18-19]. Algae play a significant role in all aquatic systems. Algae are the primary producers in an aquatic environment and sensitive enough to respond to the small changes that does not manifest observable effects on macroscopic organisms [20]. The responsds of certain species of algae to certain toxic chemicals are varying significantly [21]. Therefore, various algae are used as models for detecting the toxicity of different NPs. Some of these studies are as follows: [22] study on Al_2O_3, SiO_2, ZnO and TiO_2 in *Chlorella* sp., [23] study on TiO_2 in *Chlamydomonas reinhardtii*, [24] on Ag nanoparticle in *Pseudokirchneriella subcapitata,* and [25] on superparamagnetic iron oxide (SPION) nanoparticles in *Chlorella vulgaris.* Besides, the number of studies conducted on nano and micro B particles are rather limited. Toxicity of titanium dioxide (TiO_2), aluminium (Al) and B NPs are evaluated using *Daphnia manga*, a small crustacean that is

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extremely sensitive to the changes in the environment. B nanoparticles were classified as harmful for aquatic organisms. B ion is one of the main micro-nutrient for the normal growth and development of organisms [26-27]. On the other hand, there are a number of studies regarding B particles; such that the toxicological studies of B NPs were conducted on *Litopenaeus vannamei* (white shrimp) [28], or the development of the testicular lesions of the rats after having been treated with the boric acid [29], and the studies on determining the bioaccumulation of the B at the ppm level in human and animal tissues [27].

The objective of this study was to examine the effect of the nano and micro B particles on algae pigmentation, bioaccumulation and reactive oxygen species using *Chodatodesmus mucronulatus* which is a part of the freshwater ecosystem.

2. Materials and metods

2.1. Nanoparticles

The average size of particles are >1 μm for micro boron (micro B) particles and 50-100 nm for nano boron (nano B) particles. Boron particles were obtained from Pavezyum Advanced Chemicals (Istanbul, Turkey).

2.2. Algae culture

The test organism *Chodatodesmus mucronulatus* was collected from freshwater (Eber Lake in Afyon, Turkey), isolated and pure culture was grown according to the procedure given by Rippka, on BG-11 medium commonly used for growing blue-green algae in flasks. This medium contains trace amounts of metal ions and allows rich growth [29].

The cells were grown in sterile flasks containing 100 mL of BG-11 [29]. The cultures were grown under cool white fluorescent light (3000 lux at 25 °C), in 12 h–12 h light–dark cycle and were incubated for 15–20 days in an incubator which is suitable for photosynthesis (Figure 1). Species identification was performed manually by an expert and 18S rRNA sequencing. Ribosomal rDNA was isolated by Qiagen DNeasy Plant Kit. DNA was PCR amplified using the ITS1-ITS4 primers. Sequencing was performed by Refgen (METU, Ankara, Turkey). Phylogenetic analysis reveals that the 18s rRNA gene sequence belongs to the Chlorophyta.

2.3. Preparation of aqueous suspensions of boron particles

Stock solutions of the nano and micro B particles were prepared in deionized water. Afterwards, the solutions, after having been vortexed for 20 seconds, were sonicated with ultrasonic water bath (Bandelin, sonorex) for 40 minutes in order to increase the dispersion of the stock solutions of nano and micro B particles.

2.4. Exposure studies to acute toxicity

In order to perform B particles exposure study, 90 ml of BG-11 medium containing about 10^6 cells/ml and 10 ml of B particles solution were added into a 200 ml of conical flask. Cells were grown on an orbital shaker incubator (25°C, 85 rpm) equipped with a white light source (12h dark/light regime). After the experiment was set up, 2 ml of sample was taken after 24, 48, and 72 h to perform cell count and pigmentation measurements. The exposure study was independently repeated three times.

2.5. Determination of pigment contents

Chlorophyll *a* (C_a), Chlorophyll *b* (C_b) and carotenoids (C_c) were extracted with 80% (v/v) acetone and levels were determined according to the method of [30]. In brief, 2 ml of algae cultures were centrifuged at 2000 rpm, then the supernatant was decanted, and 80% (v/v) acetone was added and incubates dat 4°C for 24 h. Light absorption of supernatant was measured with a spectrophotometer (Hange- Lange brand DR 2800) at 663, 646 and 470 nm. C_a, C_b and C_c contents were calculated by the following formula:

$C_a=12.21A_{663}-2.81A_{646}$, $C_b=20.13A_{646}-5.03A_{663}$ and $C_c= (1000A_{470}-3.27C_a-104C_b)/229$.

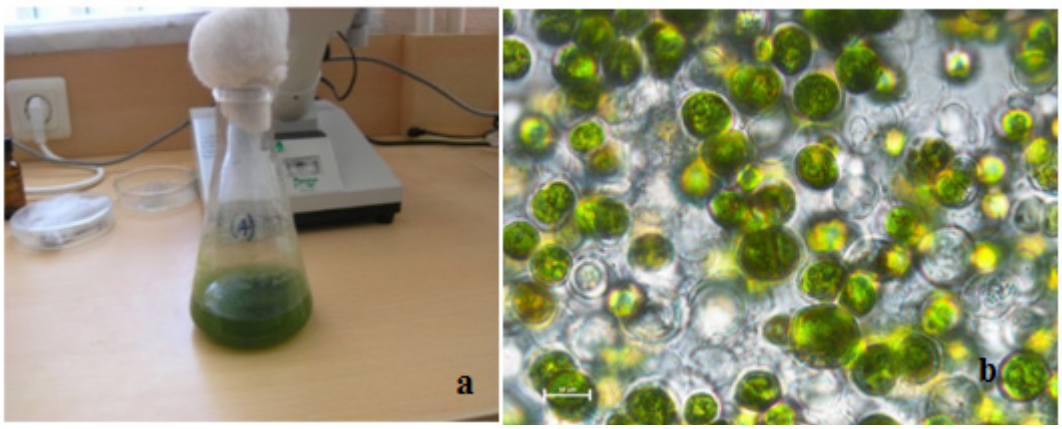

Figure 1. a.) Growth of the algae. Algae were grown in sterile culture flasks with BG-11 growth medium b.) light microscope image of *Chodatodesmus mucronulatus.*

2.6. Bioaccumulation experiments

After 72 hour exposure of *Chodatodesmus mucronulatus* to 0.1, 0.01 and 0.001 mg/L of B particles, ICP-MS (Perkin Elmer Elan DRC-e) analysis was performed to determine particle accumulation. For this process, after having been filtered (Whatman GFC/A filter) at room temperature, the algae were dried up in an oven at 105 °C for 5 h. The dried samples were put into 100 ml-beakers, and by adding 1 g of 12 ml HNO_3-$HClO_4$ (1:1) onto them, and heated in a fume hood. After the 'almost-dry' beakers had been cooled, 2 ml of concentrated $HClO_4$ was added into them, after which it was heated over a small flame until getting dried up once again. Later on, 1 g and 5 ml of concentrated HNO_3 was first added for the solution process, and after it evaporated, concentrated HCl was added and beaker was heated until it almost dried up. 1 ml of concentrated HCl was added a day later and samples were diluted to 2% HCl by bidistillated water to 25 ml, after which the samples were filtered.

EPA Method 200.7, inductively coupled Plasma-Mass Spectrometry (ICP-MS) method was utilized to measure bioaccumulation of B particles. Standard solutions for the target elements were prepared in a 2% HCl medium and fully quantitative measurements of B was performed for each sample in tripliacate [31].

2.7. Examination of cellular reactive oxygen species (ROS)

To determine toxicity behavior of B particles, intracellular reactive oxygen species (ROS) were determined. ROS are responsible for the oxidative stress associated with nanotoxicity [32]. Cellular oxidative stress levels were measured using the cell permeable indicator 20,70-dichlor-odihydro fluorescein diacetate (H_2DCFDA) [33]. Cellular esterases hydrolyze the probe to the non-fluorescent 20,70-dichlorodihydrofluorescein (H_2DCF), which is better retained in the cells. In the presence of ROS and cellular peroxidases, H_2DCF is transformed to the highly fluorescent 20,70-dichlorofluorescein (DCF). Samples were treated with 5 mM of H_2DCFDA in 1 mL of solution. The DCF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All the fluorescence data were collected using a fluorescence platereader. Samples were diluted 9 times with potassium phosphate buffer (pH, 7.8). Then, homogenization and centrifugation was performed (20 min, 10.000 rpm), the supernatant was removed.

2.8. SEM of algal cells

For the processes for SEM, after 72 h treatment with 0.1, 0.01 and 0.001 mg/L B particles, *Chodatodesmus mucronulatus* cells (about $5x10^7$ cell/ml) were collected by centrifugation at 1000 g for 10 min and 2 times. Cell pellets were incubated with 2.5% glutaraldehyde in a 0.1 M PBS overnight at +4°C. Secondary fixation were performed by adding 1% osmium tetroxide. Then, samples were washed with an ethanol solution of increasing alcohol content (from 50% to 100%). Images was taken up after sputtering with of gold (Figure 2).

2.9. Statistical analyses

All experiments were independently repeated three times and the data were recorded as the mean value with standard deviation. In order to determine the statically significant differences between control groups and treated samples by ANOVA, Tukey and single direction variance analysis; SPSS18 package was used. All data analyses were assessed based on 0.05 and 0.01 significance.

3. Results and discussion

Boron has a wide range of applications, such as semiconductors, protective coatings, domestic and industrial cleaning products, high density fuels, fiberglass, detergents, and cancer treatment [34-38]. The nanoparticle boron is considered as a potential fuel source and forms energy after metal oxidation and is used in medical research [39-40]. However boron particles reach the aquatic environment both anthropogenic and by natural means. Boron is a natural component of the boron ending aquatic ecosystem resulting from weathering of sediments and sedimentary rocks and soils [41].

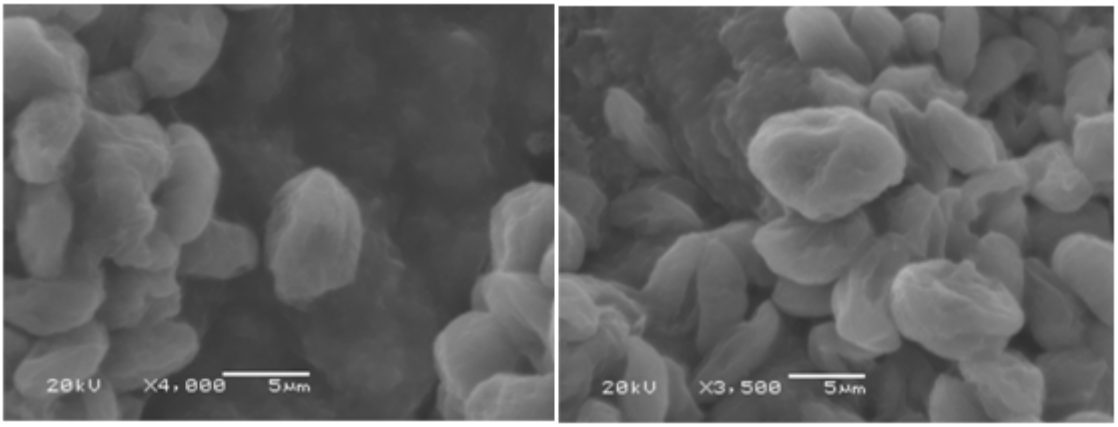

Figure 2. SEM image of *Chodatodesmus mucronulatus*

In environmental risk assessments, toxicity bioassays are made with algae to obtain information about the toxic effects of xenobiotics [42]. Because algae species differ in their different toxic chemical reactions [21]. The direct and indirect effects of nanoparticles on algae have been proven in a few studies. Direct toxic effects are mainly determined by chemical composition and surface reactivity. Because nanoparticles have a larger surface area per mass, they make them more biologically reactive, which can catalyze the redox reactions in the interaction with organic molecules [43]. In addition, photosynthesis is affected by nanoparticles because it is highly sensitive to environmental changes. Because of the accumulation of nanoparticles on photosynthetic surfaces, this can cause leaf heating and gas exchange changes due to stoma clogging [44]. The indirect effects of nanoparticles depend on various factors. The aqueous suspensions of nanoparticles are opaque and therefore shading of the light by the nanoparticles can affect overall toxicity. Therefore photosynthesis is inhibited by absorption of light by nanoparticles in algae and growth stops. Nanoparticles may serve as potential vectors due to possible interaction with other xenobiotics in the environment. Thus the suitability at the living aquatic organisms the result of this interaction may be change. For example, black carbon has a strong absorption ability and pesticide can reduce bioavailability [45-47]. It increases the membrane permeability due to the smaller size of the nanoparticles and provides maximum transmission because the cells can not properly transport the plasma membrane. This results in a significant increase in permeability causing cell death [48]. It may be toxic due to the release of toxic ions from the nanoparticles or it may have indirect effects by producing reactive oxygen species [49-50].

3.1. Effect of boron particle on pigmentation

Chodatodesmus mucronulatus was exposed to the nano and micro B particles at 0.1, 0.01 and 0.001 mg/L concentrations during 72 h.

Chlorophyll *a* (C_a) pigment content, when we compare the control group with treated groups, reduced at the 24 and 48 h, except for 72 h. Thus, we reportthat the nano B particles increased C_a amount at the first 48 h; yet, when this exposure duration was extended up to 72 h, the effect was reversed (*P>0.05*). Nano B particles has an increasing effect on Chlorophyll b (C_b) at the 24 and 48 h. However, when the exposure duration was extended up to 72 h, the nano B were seen to have a toxic effect. There is no significant change in C_b pigment levels in exposed groups in the 48 h (*P>0.05*), but, exposed groups for 72 h were suffered from the toxic effect of the nano B particles (*P<0.05*). During the 24 h exposure duration, the nano B particles had rather a toxic effect on the carotenoid (C_c) content of *Chodatodesmus mucronulatus.* There was a significant difference in terms of the effect of the nano B particles on the carotenoid content of *Chodatodesmus mucronulatus (P<0.05)* (Figure 3).

The C_a content in algae exposed to the micro B particles increased in 24, 48 and 72 h. However, there is no significant difference in exposure duration and concentration. (*P>0.05*). C_b levels were higher in the treated groups when compared to the control group and had a negative correlation with the micro B particle concentration. There is no significant difference in the exposure duration and concentration on C_b content of *Chodatodesmus mucronulatus* exposed to micro B particles (*P>0.05*). C_c levels changed sinusodally until the last day from the first day. While there was a significant difference in the effect of the micro B particles on the C_c content of *Chodatodesmus mucronulatus* in terms of the concentration (*P>0.05*), there was no significant difference in terms of time (*P<0.05*) (Figure 4).

In our study the use of two different boron particles (nano and micro-sized) led to different behaviors of boron particles in terms of toxic effects formation. Boron particles have the potential to cause toxicity in *Chodatodesmus mucronulatus* micro algae (Figure 1-2). However, algae were affected different levels from nano and micro B particles. In the nano B, the C_a, percentages at 0.1, 0.01 and 0.001 mg/L were increased 184%, 177%, 155% at 24 h and 197%, 244% and 162% at 48 h, respectively. But, when the exposure duration was extended up to 72 h, the C_a, amount decreases by 60% at 0.1 mg/L and 38% at 0.01 mg/L, whereas it increases (Figure 3). In micro B, these percentages were recorded as 156% 184% and 108% at 24 h; 136%, 135% and 94% at 48 h; and 25%, 52% and 31% at 72 h, respectively. Micro B particle effect is notable at the 3rd day of the experiment since the differece

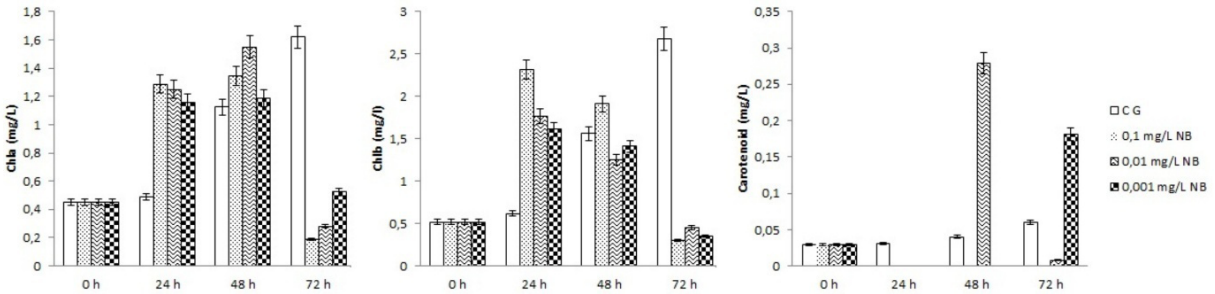

Figure 3. Chlorophyll and Carotenoid content in *Chodatodesmus mucronulatus* exposed to nano B particles for 72 h.

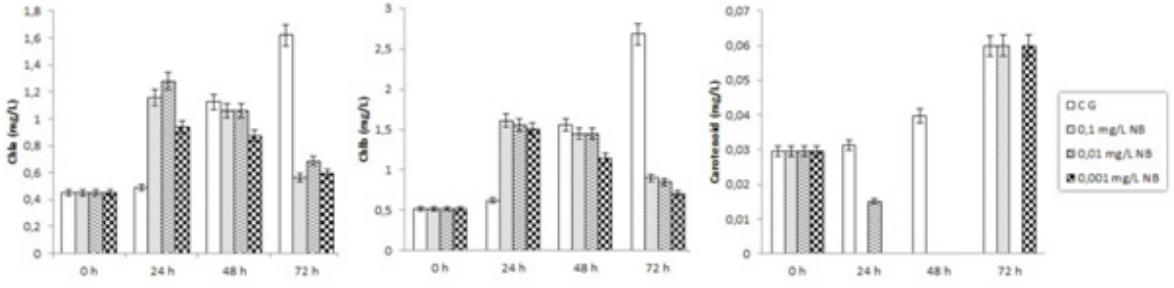

Figure 4. Chlorophyll and Carotenoid content in *Chodatodesmus mucronulatus* exposed to micro B particles for 72 h.

between control group and the treated group exceeds by 8 fold. The supportive role of the micro B on the C_a levels is concentration dependent (Figure 4). Considering the effect of the nano B particles on theC_b, pigment, it had an increasing effect in 24 and 48 h; however, the rate of increase during the 48 h exposure is rather less than that in the 24 h exposure. On the other hand, when the exposure duration was extended up to 72 h, the nano B were seen to have a toxic effect. The rates of increase are by 351%, 238% and 209% in the first 24 hours at 0.1, 0.01 and 0.001 mg/L respectively, while it is followed by 267%, 141% and 170% for 48 h; however, levels decrease by 42%, 13% and 33% at the end of 72 h. The rate of increase in 24 h at 0.1, 0.01 and 0.001 mg/L was respectively 19%, 200% and 415%, displaying that the effect of concentration of nanoparticle is rather drastic. There is no significant change in the levels of C_b pigment in the 48 h of the experiment but 72 h indicated that treated groups were suffered from toxic effect of the nano B having an approximately a quarter of the content of C_b pigments. C_b levels were higher in the micro B treated groups and C_b levels are in negative correlation with the micro B particle concentration. The rates of increase in the treated groups during 24, 48 and 72 h were 211%, 200%, 180%; 178%, 175%, 121% and 73%, 63%, 34%, respectively, for 0.1, 0.01, 0.001 mg/L. The increase rates in the control group during 24, 48 and 72 h were 19%, 200% and 415%, respectively. The effect of prolonged exposure to the micro B are proved to be harmful and leads the algae to digest some of its C_b molecule to save energy and protect itself from threats (Figure 4). During the 24 h exposure duration, the nano B had rather a toxic effect on the C_c content of algae. On the other hand, during the 48 h exposure duration, it increased by 800% at 0.01 mg/L nano B, and there was no C_c presentat 0.1 and 0.001 mg/L. When the exposure duration was extended up to 72 h, the initial C_c content decreased by 27% at 0.01 mg/L, whereas it increased by 524% at 0.001 mg/L. Considering the C_c content in the control group, it increased by 6% in the first 24 h, 34% at 48 h and 103% at 72 h. Again, considering all these results, the nano B have had a rather anomalous toxic effect on the C_c content. In particular, toxic effect was found to be acute and chronic effects were weaker (Figure 3). In micro B, C_c levels were the intriguingly change sinusodally from the first day to the second and the last. The reason could be the intervention of the secondary mechanism that at least partly nullify the effect of micro boron particles on the algae which is not properly functioning at the 72 h (Figure 4). According to these results, particle size participated in the toxicological effect. The total chlorophyll content of *Chlorella sp*. was determined after treatment with bulk and nano alumina particles. Nano and bulk the total chlorophyll content significantly lower in the exposed cells compared to that of control cells. A concentration-dependent reduction in chlorophyll content has recorded. It has also been indicated to be growth inhibitory by increasing the aluminum concentration [51]. It is likely that Ag^+ ions released from Ag nanoparticles in *Thalassiosira weissflogii* cells may reduce algae growth, photosynthesis and chlorophyll production [52].

3.2. Accumulation of boron particles

Total nano and micro B particles content determined ICP-MS analysis of *Chodatodesmus mucronulatus* is illustrated in figure 5 along with concentrations ratio. According to the statistical results, there is no difference between the nano and micro B particles in terms of accumulation (*P<0.05*). However, a significant difference in the levels of was observed between nano and micro B particles (*P<0.001*).

Green algae are currently a paraphyletic group in the kingdom of plantae and are characterized by their grass-green chloroplasts for the toxicity studies, the algae were continuously mixed in an orbital shaker at 85 rpm to provide suspension for both the particles and the algae. The toxicity studies reveal the B particles remained within the algae cells. The changes in the oxidative stress response and pigmentation, are demonstrated to be the result of the internalization of the boron particles by the algae cells (Figure 5). BG-11 medium contains trace amounts of B and negligable for this study, also shown by the ICP-MS results. High levels of bB particle in the growth medium promote internalization of B which is reduced by half in decreased concentrations. The minimum accumulation, on the other hand, was 0.01 mg/L, which was a moderate concentration. According to these data, there is no direct relationship in terms of the accumulation of the nano and micro B particles in the algae cells according to the concentration. Therefore the trend can be due

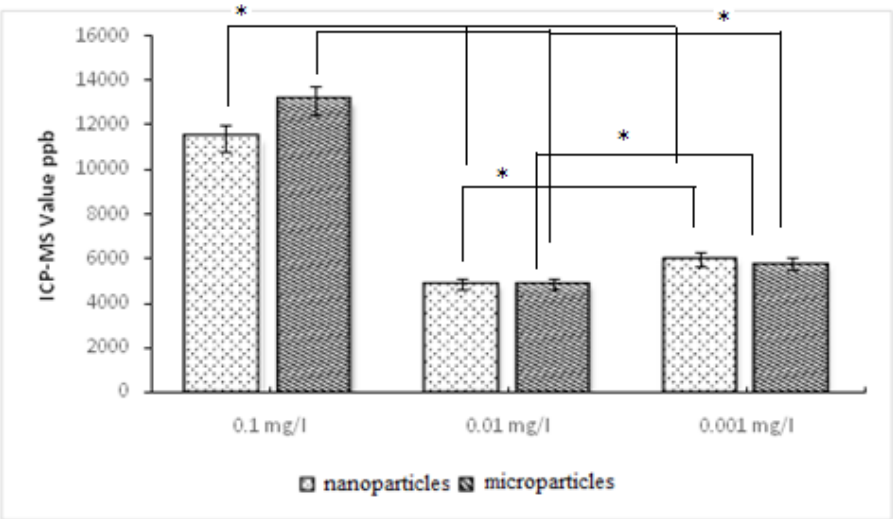

Figure 5. Bioaccumulation of nano and micro B particles in the algae cells for 72 h.

to the physico-chemical properties or the particle aggregation of the boron particles. Accumulation of boron nanoparticles have been evaluated on single cell fresh water algae *Desmodesmus multivariabilis*. According to this study, it has been observed that nano and micro boron particles accumulate in different amounts in the algae. Highest accumulation amounts, the accumulation was in 0.01 mg/L concentrations for both particles and it was measured as 6.390 ppb for nano boron, 12.490 ppb for micro boron [53].

3.3. Oxidative stres induced by suspensions of boron particles

Total cellular ROS levels were shown by flow cytometry by using H_2DCFDA, an afluorogenic ROS indicator. Exposure of *Chodatodesmus mucronulatus* to nano and micro boron particles induced an increase in the algae cell ROS levels. A significant toxicity occurred in the algae cells in all conditions after 72 h exposure (*P<0.01*). The toxicity was pronounced the most between low and moderate boron exposure. When we compared the control group with the exposed groups, the ROS levels in the algae cells was increased substantially in low and moderate nano boron particles exposure, whereas it diminished at 0.1 mg/L (*p0.05*). When we compared the exposed groups boron particles among themselves, the nano boron particles were reported to have been more toxic than the micro boron (*p0.05*) (Table 1).

Nanoparticles can cause indirect toxic effects by forming reactive oxygen species (ROS). ROS levels are an indication of an oxidative stress associated with nanotoxicity [54-55]. The results of this study indicate that sensitivity to acute toxicity in algae varies depending on particle size. In order to verify whether or not it is effective on algae in the same way, ROS levels in the algae cells were compared between the control group and the treated groups. There was some toxicity observed in the micro B particles treated group behaving differently since the ROS level increased at low and moderate micro B particle exposures. The highest ROS level was reported to have been seen in 0.1 mg/L micro B. The ROS levels increased along with the increasing concentration in the micro B particles which is expected; however, no similar relationship was observed in the nano B particle exposure. The ROS level recorded at the highest concentration (0.1 mg/L) is even lower than that of the control group. Interestingly, the ROS level rose in the decreasing concentration of the nano B, which exhibit that the algae may act unexpectedly to the nutrients in the form of nanoparticles. Moreover, It was also noted that both boron particles had different effects on cellular toxicity and were reported to be statistically significant (*P<0.05*). Considering the results of ROS analysis, the boron particles pose an oxidative stress on the *Chodatodesmus mucronulatus*. The toxicity of the nano B is more than the micro B; separately, there is toxicity in a different way in each concentration of the nano B particles, since each concentration caused a different ROS level. In the highest concentration, there was less ROS level from the control group.

Table 1 Oxidative stres levels associated with exposure to the suspensions boron particles

Concentration (mg/L)	Boron Nanoparticles	Boron Microparticles
0	100±0.00	100±0.00
0.001	151.66±0.57	101.33±0.57
0.01	160.66±0.57	123.66±0.57
0.1	88.01±0.00	130.66±15.50

Values are mean ± standart deviation for ROS concentration (ppb) in *Chodatodesmus mucronulatus*after 72-h exposures

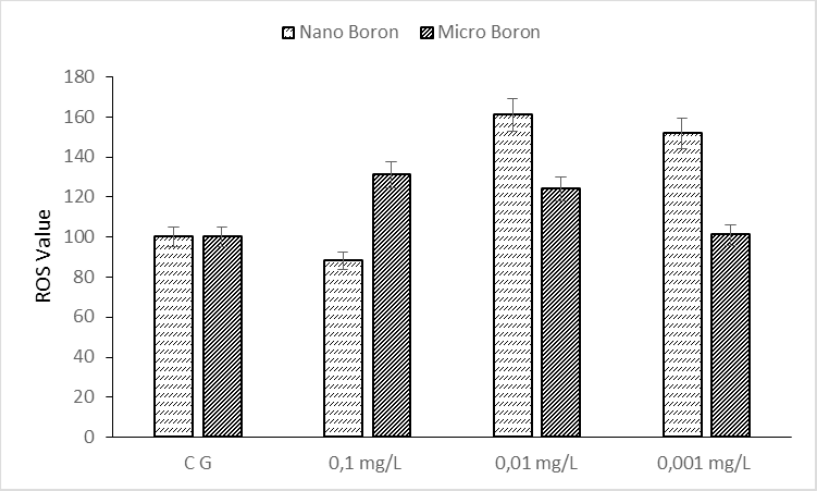

Figure 6. The ROS values of the boron particles within the *Chodatodesmus mucronulatus* after 72 hours.

4. Conclusion

This study evaluated the acute toxicity of nano and micro B particles to fresh water algae *Chodatodesmus mucronulatus*. We were assessed in the sensitivity of the tested algae to micro and nano B particles, documenting the effects of sizes and concentrations form of B on the toxicity results. The data obtained from our study showed that nano and micro boron particles have varying degrees of effects on the pigment content, ROS and deposition of the plant during varying periods of duration. Generally boron nanoparticulate exhibited positive effect on the plant.

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